Journal of Pharmaceutical and Biomedical Analysis
30 (2003) 1725–1734

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Review

# New label enzymes for bioluminescent enzyme immunoassay

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Received 7 May 2002; received in revised form 12 August 2002; accepted 17 August 2002

Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

**Abstract**

Firefly luciferase catalyzes the oxidation of luciferin in the presence of ATP, magnesium ion and molecular oxygen with a high quantum yield. Due to its high sensitivity and specificity for ATP, luciferase has been used for bioluminescent detection of ATP in various biological samples. But it is not known well to apply the detection of immunoassay. In this article, the use of various enzymes as labels in the design and development of immunoassays for biomolecules has been reviewed.

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*Keywords:* New label enzymes; Bioluminescent enzyme immunoassay; Firefly luciferase; Acetate kinase (AK); Pyruvate phosphate dikinase (PPDK)

## 1. Introduction

Recently, bioluminescence has been extensively investigated by several researchers for its high sensitivity with the aim of applying several assays [1], and the various luciferases and photoprotein genes were cloned and expressed in *Escherichia coli* or *Saccharomyces cerevisiae* [2–4]. Especially, recombinant firefly luciferase is now commercially available and has been employed for enzyme immunoassay (EIA). However, firefly luciferase loses much of its activity when treated with the chemical cross-linkage reagents used in the conjugation. To solve this problem, Miska et al. synthesized D-luciferin-*O*-phosphate and other luciferin derivatives, which did not react with luciferase, which will react as substrates for alternative enzymes. Also we have established two highly sensitive bioluminescent assays for thermostable acetate kinase (AK) or pyruvate phosphate dikinase (PPDK). After EIA using AK or PPDK as label enzyme, AK or PPDK activity was determined by measuring the amount of produced ATP using firefly luciferase [5,15]. Furthermore, we have introduced a well-known streptavidin–biotin system to EIA using biotinylated AK [6,7]. Using these systems, we applied to assay for biologically active substances such as hCG, mIL-6, insulin and other clinically important substances. On the other hand, Hukuda et al. have

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PII: S0731-7085(02)00514-9

developed bioluminescent EIA (BLEIA) with biotinylated firefly luciferase [8]. Seto et al. developed BLEIA for TSH and prostate-specific antigen (PSA) using biotinylated firefly luciferase [16,17]. More recently, Kajiyama et al. [9] conducted mutational analysis of firefly luciferase and showed that they emit light of different colors due to single amino acid change. By using these two kinds of biotinylated thermostable luciferase (*Luciola lateralis*), Ohkuma et al. [10] developed a simultaneous EIA of pepsinogen (PG) I and II in serum.

## 2. Bioluminescent EIA using AK as a label enzyme

We have developed the first sensitive BLEIA that utilizes AK labeled antibody or antigen coupled with the firefly luciferase. The bioluminescent assay of AK was done by firefly luciferase for measuring ATP produced by enzymatic reaction of AK using acetyl phosphate and ADP as substrate. As the reaction principle is shown in Fig. 1, ATP produced by AK was determined by luciferin and recombinant firefly luciferase. AK was conjugated to 17α-hydroxyprogesterone (17-OHP), human thyroid stimulating hormone (TSH) or anti-rabbit IgG antibody Fab′ fraction and rabbit IgG. The immunoassay procedures used a heterogeneous method for bound and free separation. In AK assay, various factors affecting light intensity, such as concentrations of substrate, the kind of buffer and its concentration or pH, reaction temperature and time, were examined. Enzymatic and immunoreactivity of AK conjugated hapten and antibody also affected the sensitivity of BLEIA. Therefore, we examined

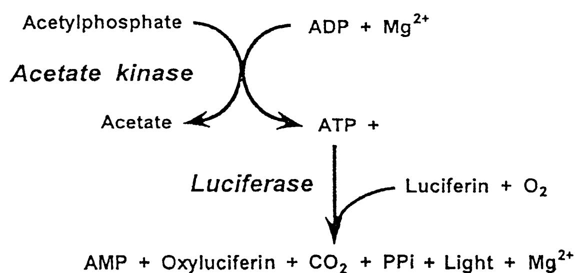


Fig. 1. Principle of bioluminescent assay for AK.

the combination of dilution ratio of antibody and AK-conjugated hapten or antibody. The conditions were selected based on the results of optimization study and AK substrates were optimized for maximal sensitivity and specificity.

Endpoint assay and rate assay of AK activity were performed. Under the optimal assay conditions, the linearity of both standard curves ranged from $10^{-20}$ to $10^{-16}$ mol/assay for AK are shown in Fig. 2. In the endpoint assay, the coefficient of variation (CV%, $n = 5$) for each point was 2.1–8.3%. In the rate assay, the CV (%, $n = 6$) ranged from 0.61 to 3.5. The detection limits (at blank + 2S.D.) of endpoint and rate assay were $1.4 \times 10^{-20}$ and $6.5 \times 10^{-20}$ mol/assay, respectively. The comparison of two AK assay methods is

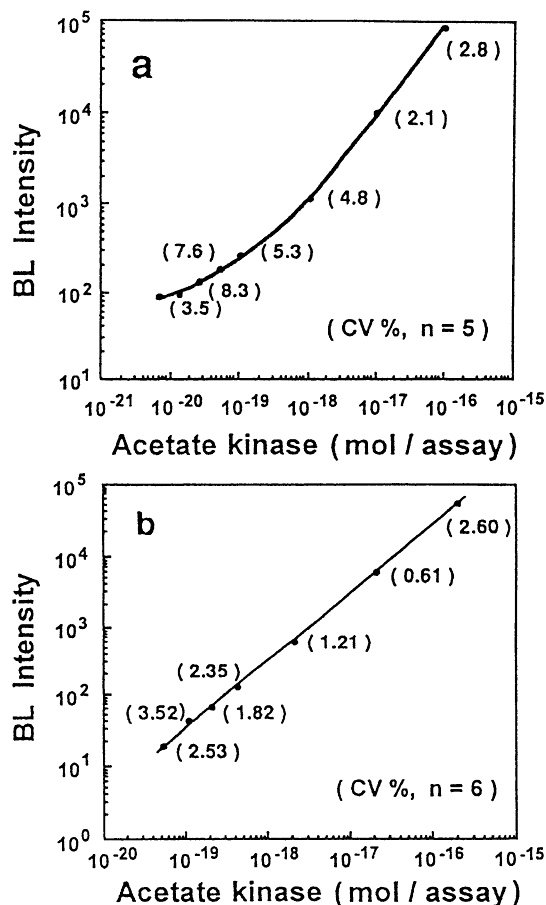


Fig. 2. Standard curve for AK by endpoint (a) and rate assay (b).

Table 1
Comparison of endpoint and rate assay of acetate kinase

| Assay method | Sensitivity (mol/assay) | Reaction or lag time (min) | Total assay time (min/60 samples) |
|---|---|---|---|
| Endpoint assay | $1.4 \times 10^{-20}$ | 60 | 90 |
| Rate assay | $6.5 \times 10^{-20}$ | 15 | 16 |

shown in Table 1. The endpoint assay is more sensitive than the rate assay, but more time consuming. In the rate assay, the enzyme reaction is initiated by adding only one reagent and bioluminescent intensity measured. Thus rate assay of AK is simple and useful for various EIAs. The sensitivity of this method is almost the same as the sensitive chemiluminescence assay of alkaline phosphatase using AMPPD [11,12] as substrate and higher than the bioluminescent assay of alkaline phosphatase using D-luciferin-*O*-phosphate as the substrate.

The measurable range and detection limit of 17-OHP, TSH and rabbit IgG are shown in Table 2, the measurable range of 17-OHP was from 0.1 to 50 pg/assay and the detection limit was 0.1 pg, which corresponds to 300 amol/assay. The reproducibility of each point of standard curve ranged from 1.3 to 5.5% ($n = 5$). The measurable range of TSH was 0.006–45 μIU/ml and the accuracy of within assay was 1.72–7.14% ($n = 6$) using standard serum of TSH. The standard curve of rabbit IgG was 0.01–25 ng/assay, and the detection limit was 12.5 pg/assay, which corresponds to 83 amol/assay calculated using the value of 150 000 as molecular weight of rabbit IgG.

The TSH values of same samples were assayed by fluorometric EIA used routinely (AIA 1200, Tosoh) and the proposed BLEIA, and the result is shown in Fig. 3.

Table 2
2. Comparison of 17-OHP, TSH, and rabbit IgG BLEIAs

| Assay | Measurable range | Detection limit |
|---|---|---|
| 17-OHP | 0.1–50 pg/assay | 0.1 pg (300 amol)/assay |
| TSH | 0.006–45 pIU/ml | – |
| Rabbit IgG | 0.01–25 ng/assay | 12.5 pg (83 amol)/assay |

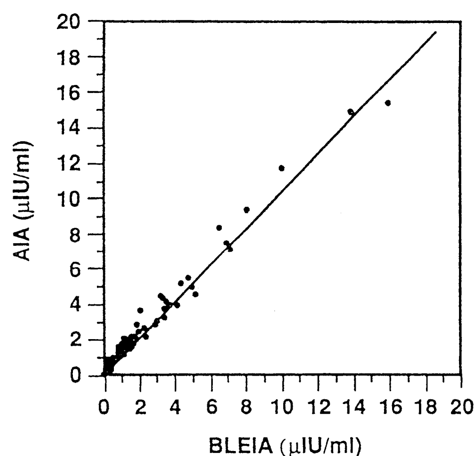


Fig. 3. Correlation between BLEIA and AIA.

The correlation between TSH values obtained by both methods were extremely satisfactory: $y$ (AIA) $= 1.04x$(BLEIA)$+0.16$, $r = 0.989$ ($n = 82$). In addition, seven samples that were below the detection limit of conventional fluorimetric EIA were measured by the proposed BLEIA. The method mentioned here is the first application of AK labeled antigen or antibody coupled with the firefly luciferase–luciferin system to the development of very sensitive BLEIA. Especially, in the

Table 3
Reproducibility of the TSH BLEIA

| TSH concentration (μIU/ml) | CV% ($n = 6$) |
|---|---|
| 0 | 1.72 |
| 0.006 | 4.06 |
| 0.013 | 3.99 |
| 0.025 | 2.51 |
| 0.05 | 3.35 |
| 0.1 | 1.79 |
| 1.0 | 3.99 |
| 10 | 7.14 |

TSH assay, sensitivity was about 25 times higher than that of radioimmunoassay (RIA) and it is extremely reproducible (Table 3). It will be useful for the detection of the low concentration of TSH values seen clinically in conditions such as hyperthyroidism resulting from Grave's disease or thyroid tumor. Differently from conventional chemiluminescence immunoassays, the system mentioned here has the advantage that decay of light is absent. This is due to the light generation system by AK and luciferase. The use of solid-phase coated with second antibody, to indirectly immobilized anti-TSH or anti-rabbit IgG antibody and the separation step allows the removal of potential interference from serum components.

To produce universal reagent for the AK-liked BLEIA system, we prepared biotinylated AK. Streptavidin (SA), biotin-binding protein produced by *Streptmyces avidinii* has an exceptionally high binding affinity ($10^{-15}$ M) for biotin known as vitamin H and a coenzyme for enzymes involved in carboxylation reaction. Because of this high affinity, early investigators explored the use of the streptavidin–biotin system in immunoassays. These systems provide excellent sensitivities in immunoassay. Furthermore, the application of various biotinylating reagents contributes to the standardization in sandwich type immunoassays since it employs only one labeled substance for the detection of various antigens. Biotinylated polyclonal or monoclonal antibodies, used as primary probes, are prepared under mild conditions with commercially available *N*-hydroxysuccinimide ester or maleimide derivatives of biotin. The SA-biotin based BLEIA procedure and standard curve for human chorionic gonadotropin (hCG) was shown in Fig. 4; the measurable range was 0.003–2.0 mIU/ml. Intra-assay CVs ranged from 3.8 to 9.8% corresponding to 0.003–2.0 mIU/ml. The hCG values of serum samples were assayed by the BLEIA and time resolved fluorescent immunoassay (DELFIA, Pharmacia, Finland).

As shown in Fig. 5, the correlation between results obtained by both methods was extremely satisfactory, $y$ (BLEIA) $= 0.951x$(DELFIA) $+$ 1.58, $r = 0.994$ ($n = 50$). In addition, the BLEIA was capable of measuring 11 samples that were below the detection limit of the DELFIA.

Generally, small animals such as rats or mice are used in experiments for developing new drugs. However, these animals are not able to provide a large quantity of samples such as biological fluid, tissues and plasma. Therefore, a sensitive assay method is required to measure extremely low levels of target compounds contained in a small quantity of samples in many investigations. The mouse interluekin-6 (mIL-6) was measured by the SA-biotin based BLEIA as the example. The measurable range of mIL-6 was 7.8–4000 pg/ml and the detection limit (blank $\pm$2S.D.) was 6.6 pg/ml. The detection limit of mIL-6 obtained by BLEIA was 6-fold higher than that obtained by colorimetric EIA, which used the same antibodies and was carried out by a similar immunoreaction method. The recovery test and sample dilution test for mIL-6 were performed by using mouse tissue extract samples spiked with different levels of mIL-6 (0, 125, 500, 2000 pg/ml) (Table 4). For sample dilution test, four samples of mouse tissue extracts, which were from brain, kidney and mesentery, were serially diluted (until 1:64) with an immunoreaction buffer and measured. Good linearity was observed with each sample concerning the relation of the extent of dilution and assay result. Recently, Takaki et al. reported that the plasma concentration of the cytokine IL-6 levels is increased by non-inflammation stress, such as immobilization [13]. Therefore, we developed a BLEIA to measure mIL-6 level in various tissues from normal control mice, mice stressed by immobilization for 1 h followed by a 1-h rest, and mice treated with the inflammatory stimulus lipopolysaccharide (LPS, 1 mg/kg injected i.p. 2 h prior to sacrifice). The IL-6 levels of the LPS-treated mice were significantly higher than those of control group, as would be expected (Table 5). The stressed mice displayed higher levels of mIL-6 in the liver and plasma and lower mIL-6 levels in the brain and kidney than the control group did.

Pituitary adenylate cyclase activating polypeptide 38 (PACAP 38), originally isolated from ovine hypothalamus, is a novel peptide hormone and has a variety of biological actions. In studies of the physiological behavior of endogenous PACAP, the determination of PACAP38 levels in biological materials requires a highly sensitive and specific

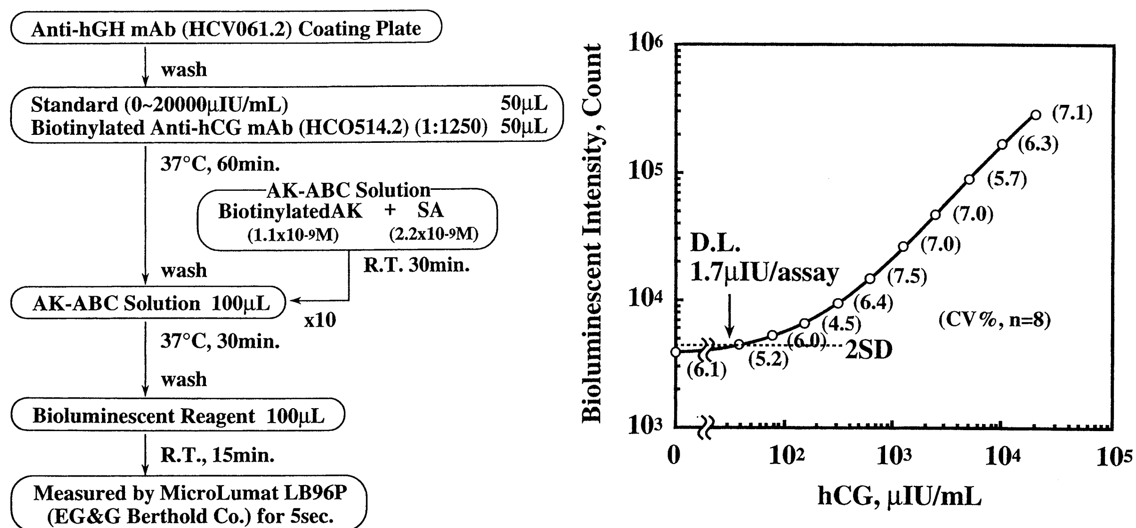


Fig. 4. Assay procedure and standard curve for hCG.

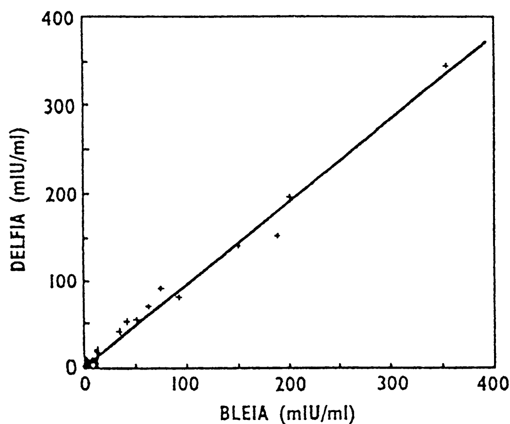


Fig. 5. Correlation between BLEIA and DELFIA.

Table 4
Recovery of mIL-6 from plasma and tissue extract

| | Amount added to sample (pg/ml) | Percent recovery |
|---|---|---|
| Plasma | 31, 125, 500 | 85.2 ± 17.6 ($n = 6$) |
| Tissue extract | 0, 125, 500, 2000 | 84.3 ± 15.3 ($n = 12$) |

method. The BLEIA uses biotinylated PACAP38 as a labeled antigen and biotinylated AK–strepavidin complex bound to a solid phase as a detection system. The measurable range was 62–16 000 pg/ml for PACAP38; its concentration of various tissue extracts and rat and human plasma could be measured by the proposed BLEIA. The PACAP38 concentrations were also in various tissues of SD strain rats, which were perfused or unperfused, measured (Table 6). The highest concentration of PACAP38 was in the hypothalamus, and other brain areas also showed high concentrations. The testis contained the highest level of PACAP38 among those found in the peripheral tissues. When rat was perfused, the PACAP38 concentrations decreased in all tissues. Perfusion decreased the amount of PACAP38 seen in all tissues, suggesting that PACAP38 circulates with the blood. These results suggested that considerable amounts of PACAP38 existed in the blood. The plasma levels of PACAP38 measured in SD rats and humans were 265.5 ± 82.6 ($n = 6$) and 596.4 ± 180.6 pg/ml ($n = 40$), respectively.

Table 5
Tissue concentrations of mIL-6 in LPS-treated, stressed, and normal mice

| Tissue | Control | Immobilization-stressed | LPS-treated |
|---|---|---|---|
| Brain | 7.25±0.8 | 5.14±1.2 | 14.06±1.5 |
| Heart | 5.47±2.8 | 3.41±0.9 | N.D. |
| Lung | 4.98±1.2 | 4.58±1.4 | N.D. |
| Stomach | 9.88±6.8 | 6.20±2.0 | N.D. |
| Duodenum | < 0.08 | 0.70±1.2 | N.D. |
| Jejunum | 2.56±2.8 | 1.78±1.6 | N.D. |
| Ileum | 4.36±0.9 | 2.74±0.8 | N.D. |
| Colon | 1.89±0.4 | 2.15±0.2 | N.D. |
| Mesentery | 50.94±21.7 | 56.56±33.8 | 160.59±75.5 |
| Liver | 146.37±17.2 | 200.77±27.2 | 165.69±12.4 |
| Spleen | 2.53±0.5 | 4.79±4.1 | 269.76±105.8 |
| Kidney | 105.03±7.0 | 78.64±36.3 | 370.35±63.4 |
| Muscle | 3.24±1.2 | 4.47±3.5 | N.D. |
| Plasma | Below detection limit | 148.8±53.2 | 125.6±73.7 |

N.D., not determined.

Table 6
Tissue concentrations of PACAP in SD rats (ng/g wet tissue)

| Tissue/brain region | With perfusion | Without perfusion |
|---|---|---|
| Hypothalamus | 207.00±41.13 | 427.3±92.7 |
| Cortex | 11.06±0.225 | 42.08±3.37 |
| Hippocampus | 13.72±6.19 | 61.56±8.54 |
| Anterior pituitary | 8.56 | – |
| Posterior pituitary | 46.45 | – |
| Lung | 1.67±0.61 | 3.83±0.26 |
| Atrium | 1.75±0.38 | 4.90±0.99 |
| Liver | 1.53±0.63 | 5.82±1.08 |
| Spleen | 3.04±0.87 | 9.90±1.88 |
| Pancreas | 1.19±0.47 | 9.71±0.87 |
| Stomach | 5.11±1.53 | 12.88±2.22 |
| Duodenum | 5.88±1.39 | 19.84±2.33 |
| Jejunum | 4.12±0.17 | 23.13±5.57 |
| Ileum | 2.82±1.27 | 23.48±4.96 |
| Colon | 2.88±1.10 | 24.63±6.01 |
| Kidney | 1.54±0.71 | 6.79±2.9 |
| Adrenal gland | 4.75±2.30 | 16.70±2.68 |
| Testis | 51.32±5.46 | 66.15±9.75 |
| Epididymis | 1.20±0.17 | 13.00±2.75 |
| Ovary | 1.53±0.22 | 17.42±5.8 |

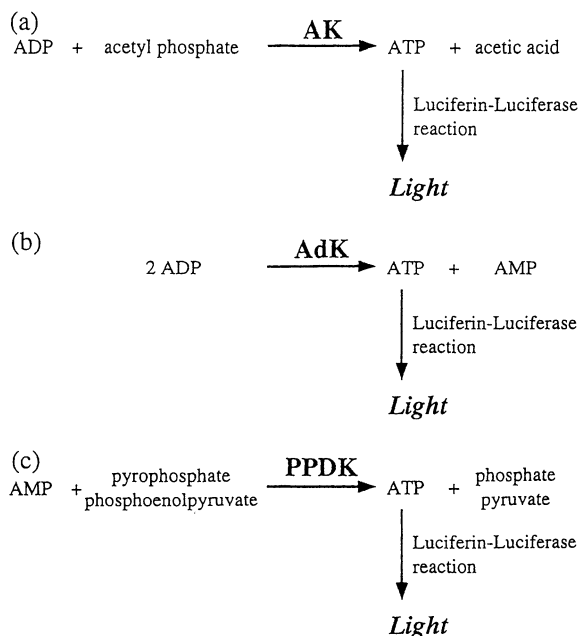


Fig. 6. Bioluminescent reaction for (a) AK; (b) AdK and (c) PPDK.

## 3. Bioluminescent EIA using PPDK as a label [15]

PPDK catalyzes the formation of ATP from AMP, ppi (diphosphate) and phosphoenolpyruvate (PEP) (Fig. 6c). The BL assay of PPDK was highly sensitive with a low background because it was not affected by adenylate kinase (AdK), which generates ATP from 2mol of ADP and is present in various microorganisms. Since the BL assay of AK requires ADP as a substrate (Fig. 6a), AdK increases the background luminescence, and as a

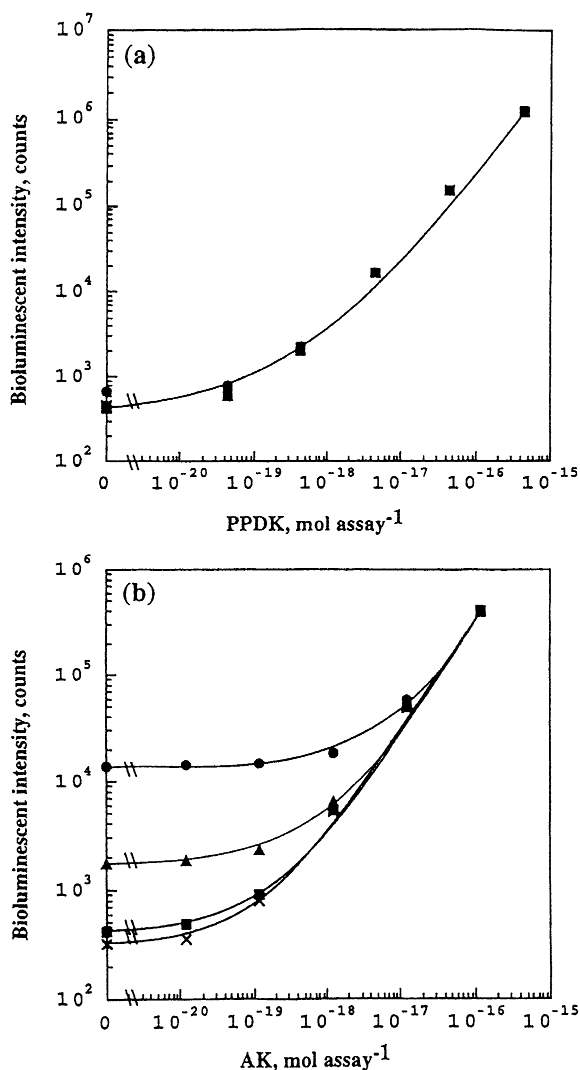


Fig. 7. Influence of AdK on bioluminescence assay of (a) PPDK and (b) AK. The amounts of AdK are: (●) 10; (■) 1; (▲) 0.1 μU per assay; (×) control.

result, detectability of AK decreases. We speculated the BL assay for PPDK in the presence of contamination of AdK would achieve greater detectability because of the low background, because ADP is not needed for the enzymatic reaction of PPDK. Fig. 7 a shows the influence of AdK on luminescence assay of PPDK and AK. In the PPDK assay, there was no increase in background luminescence on addition of various concentrations of AdK, however, the AK assay was

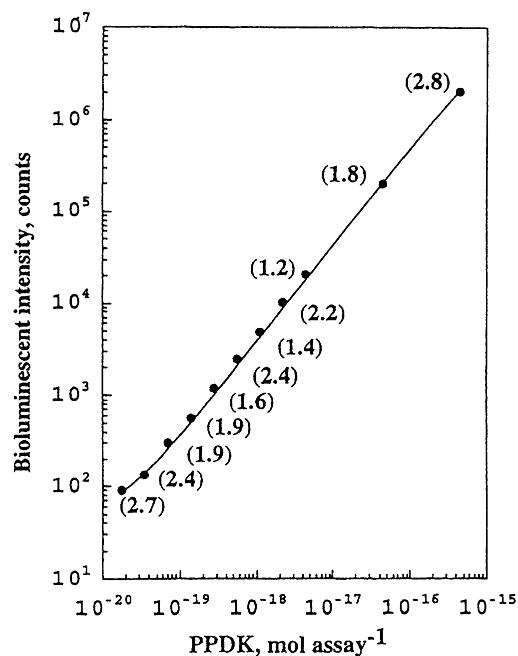


Fig. 8. Calibration graph for PPDK using the proposed bioluminescent assay. Values in parentheses are the intra-assay coefficient of variation (%, $n = 8$).

shown to be affected by addition of AdK, and delectability for AK was decreased according to the increase in AdK concentration.

As shown in Fig. 8a, the calibration curve of the assay ranged from $3.5 \times 10^{-20}$ to $4.5 \times 10^{-16}$ mol/assay. The intra-assay coefficient of validation (CVs) for eight replicates with each calibration point of PPDK was from 1.2 to 2.8% as shown in Fig. 8. The detection limit (blank + 3S.D.) was $1.36 \times 10^{-20}$ mol/assay. The BL intensity was stable for 120 min at 37 °C (Fig. 8b).

In the proposed BLEIAs for alpha-fetoprotein (AFP) and insulin, we used PPDK-labeled rabbit anti-FITC Fab′ as enzyme labeled antibody (Fig. 9). Fig. 10 shows calibration graphs for AFP and insulin, whose measurable range of AFP and insulin were 0.04–208 ng/ml and 0.63–40.4 μU/ml, the detection limits of AFP and insulin were $2.8 \times 10^{-18}$ and $9.3 \times 10^{-17}$ mol/assay, respectively. The intra-assay CVs of AFP and insulin at each point were 2.3–6.1% ($n = 6$) and 1.3–5.7% ($n = 8$), respectively. The inter-assay CVs for nine replicates with 3.51, 22.28, and 65.33 ng/ml AFP in

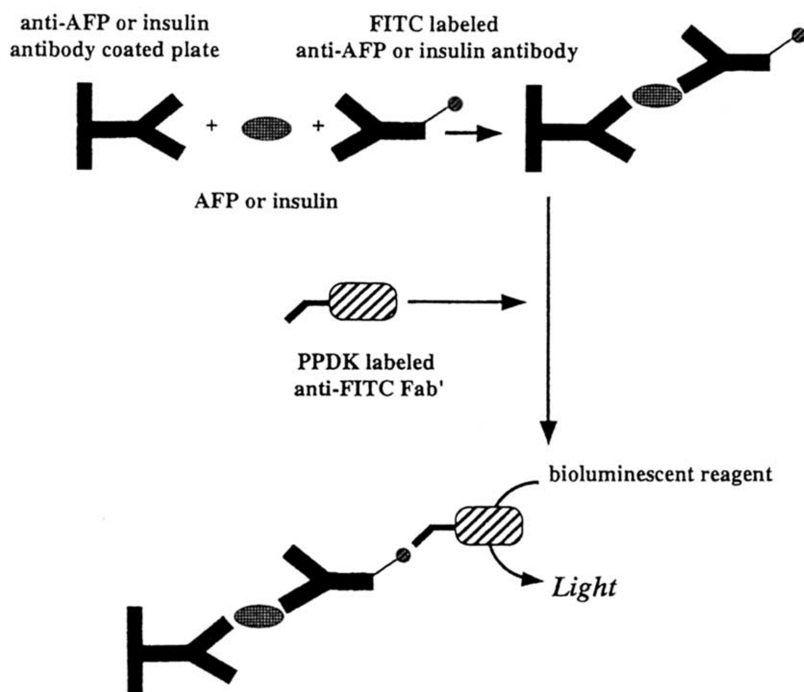


Fig. 9. Schematic illustration of the proposed BLEIA. FITC: fluoresceinisothiocyanate.

plasma samples were 4.3, 3.5, and 4.5%, and with 8.57 and 17.07 μU/ml insulin in the plasma sample were 8.1 and 7.0%, respectively.

Recovery and sample dilution test for AFP and insulin were carried out on human plasma samples. In the recovery test, various concentration of AFP or insulin in plasma sample were spiked, and then determined by proposed BLEIA. The recovery of AFP from plasma samples was $87.5 \pm 13.8\%$ (mean $\pm$ S.D., $n = 20$) and insulin was $82.9 \pm 12.7\%$(mean $\pm$ S.D., $n = 20$), respectively. For the sample dilution test, good linearity was observed until the dilution ratio of 1:62. The AFP and insulin concentrations of plasma samples were measured by the proposed BL-EIA and conventional methods. The methods that were compared were electrochemiluminescent immunoassay (ECLIA, Roche Diagnostics Co. Tokyo) for AFP and fluorescent EIA (Tosoh Co., Tokyo) for insulin. These measurements were carried out in the clinical laboratory, Showa University Hospital. The regression equations were $y$ (BLEIA) $= 1.046x$(ECLIA)$-0.617$ ($n = 94$, $r = 0.999$) for AFP and $y$ (BL-EIA) $= 0.735x$(Fluorescent EIA)$-0.082$ ($n = 105$, $r = 0.970$) for insulin.

## 4. Bioluminescent EIA using biotinylated firefly luciferase as a label

The application of luciferase–luciferin reaction is to be expected in the field of clinical diagnostics because of its high quantum yield. However the practical application of luciferase for clinical diagnostics has not yet succeeded well due to its insufficient stability. Recently, a method to biotinylate proteins by means of genetic engineering became available. Biotin enzymes such as acetyl-CoA carboxylase and pyruvate carboxylase contain a biotin molecule, which is covalently attached to a unique Lys via an amino linkage catalyzed by biotin holoenzyme synthetase. Tatsumi and Hukuda produced biotinylated luciferase consisting of *Luciola lateralis* luciferase fused to either an artificial biotin acceptor peptide or the carboxyl-terminal 87 residues of *E. coli* biotin carboxyl

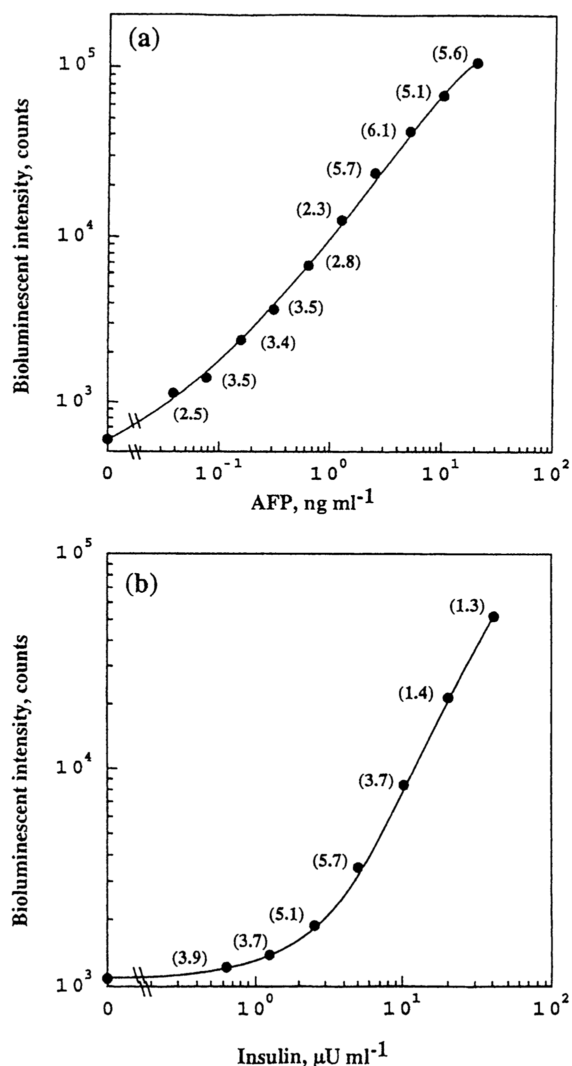


Fig. 10. Calibration graph for (a) AFP and (b) insulin using the proposed BL-EIA. Values in parentheses are the intra-assay coefficient of variation (%, $n = 8$).

carrier protein [14]. Therefore, sensitive BLEIAs of hCG, human growth hormone (hGH), TSH, PSA [6–17] and staphylococcal protein A (SPA) have been developed using biotinylated firefly luciferase in combination with streptavidin and biotinylated antibodies. Furthermore, a color mutation could be introduced luciferase by site-directed mutagenesis. Using these biotinylated luciferases that emit light of different colors, Fukuda et al. demonstrated a simultaneous detection of mouse IgG, and mouse IgM in immunoblotting [8]. We then employed these mutants in a simultaneous BLEIA of PGI and PGII in serum. PGI and PGII are inactive zymogens of pepsins, the proteolytic enzymes found in gastric secretions. Serum levels of PGI and PGII and the ratio PGI/PGII are used to evaluate gastric atrophy, gastric ulcer, and gastric acidity. Because lesions with a great degree of gastric atrophy are associated with a high incidence of stomach cancer, the determination of serum PGI and PGII may be useful in the screening of population at high risk of stomach cancer. A simultaneous assay of PGI and PGII in serum would be a cost-effective and laborsaving method of performing this screening. The principle of simultaneous BLEIA of PGI and PGII in serum is shown in Fig. 11. Magnetic particles coated with anti-PGI and anti-PGII antibodies were used to capture serum PGI and PGII. A triplex (stable complex of the following three components, namely) of biotinylated anti-PGI or anti-PGII antibodies, streptavidin, and biotinylated luciferase mutant was then added. After bound/free separation by washing, the bound enzyme activities of the two luciferase were measured at $\lambda_{max} =$ 670 nm (PGI) and $\lambda_{max} = 559$ nm (PGII).

The value of PGI was obtained by calculation based on the bioluminescent intensity at $\lambda_{max} =$ 607 nm. The value of PGII ($\lambda_{max} = 559$ nm) was obtained by the method using a band pass filter. The calibration range of PGI was from 2 to 200 ng/ml, and that of PGII was from 1 to 100 ng/ml.

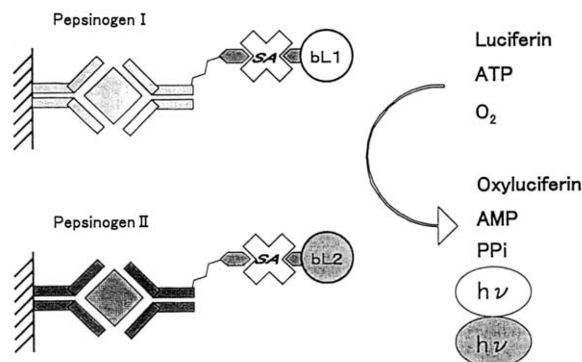


Fig. 11. Principle of simultaneous assay of PGs. SA: Streptavidin, bL-1: biotinylated luciferase 1, bL-2: biotinylated luciferase 2.

Table 7
Simultaneous BLEIA of PGI and PGII in serum

| | CV (%) | Comparison with single assay |
|---|---|---|
| PGI | 3.4–10.2 | $y$ (simultaneous assay) = $0.995x$ (ELISA)+0.84, $r = 0.948$ |
| PGII | 4.0–8.6 | $y$ (ELISA) = $1.055x$ (simultaneous assay)+1.47, $r = 0.974$ |

Both assays are highly reproducible; the values obtained by the simultaneous assay correlate well with the value obtained by single assay (Table 7). Thus, the proposed simultaneous BL-EIA of PGI and PGII has many features that would make it a useful clinical tool.

## 5. Conclusion

Two types of highly sensitive bioluminescent assays of AK have been developed using firefly luciferase to determine the amount of ATP produced by AK. AK labeled antigen or antibody was used for the development of very sensitive BLEIA. Especially in the TSH assay, sensitivity was about 25 times sensitive standard RIAs, and do not exhibit the decay of light effect seen in conventional chemiluminescent immunoassays. It will be useful for detection of low concentrations of TSH values such as the serum levels in patients with hyperthyroidism. It has been applied to BLEIA in combination with the streptavidin–biotin system with biotinylated AK and appeared to be sensitive, simple, and convenient. Using the streptavidin–biotin system, mIL-6 in mouse tissue and plasma, and PACAP 38 in rat tissues could be accurately measured without the concentration of samples or any other pretreatment using the BLEIA. In order to decrease background luminescence from contamination of AdK, PPDK has been developed. The BL assay for PPDK was highly sensitive with a low background because it was not affected AdK. We also developed BLEIA for AFP and insulin using PPDK as label enzyme. The study demonstrated that simultaneous detection of two antigens is achievable using the two kinds of biotinylated luciferase–streptavidin–biotinylated antibody complexes that were capable of emitting different colors of light. As a clinical application using this system, PGI and PGII in serum can be satisfactorily assayed. Further investigation is now ongoing to determine if multiple analytes in the same sample can be assayed by BLEIA.

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